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**The blood-brain barrier in multiple sclerosis:**

**microRNAs as key regulators**

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**Abstract**

Multiple sclerosis (MS) is a progressive inflammatory disease of the central nervous system (CNS) leading to severe neurological deficits. To date, no treatment is available that halts disease progression, but clinical symptoms can be generally improved by therapies involving anti-inflammatory and/or immune modulatory reagents, which may cause off-target effects. Therefore, there remains a high and unmet need for more selective treatment strategies in MS.

An early event in MS is a diminished function of the blood-brain barrier (BBB) which consist of specialized brain endothelial cells (BECs) that are supported in their barrier function by surrounding glial cells.

Leakage and inflammation of the BECs in MS patients facilitate the massive influx of leukocytes into the brain parenchyma, which in turn induces irreversible demyelination, tissue damage and axonal dysfunction. Identification of ways to restore BBB function and promote its immune quiescence may therefore lead to the development of novel therapeutic regimes that not only specifically reduce leukocyte entry into the central nervous system but also restore the disturbed brain homeostasis. However, the complex network of molecular players that leads to BBB dysfunction in MS is yet to be fully elucidated. Recent discoveries unravelled a critical role for microRNAs (miRNAs) in controlling the function of the barrier endothelium in the brain. Here

we will review the current knowledge on the involvement of BBB dysfunction in MS and the central role that miRNAs play in maintaining BBB integrity under inflammatory conditions.

**Keywords**

Biomarkers, blood-brain barrier, exosomes, inflammation, microRNA, multiple sclerosis, therapeutic strategies

## *1. Multiple Sclerosis (MS), the disease and its characteristics*

### 1.1. MS: etiology and clinical course

MS is a progressive disease of the central nervous system (CNS) with an autoimmune component. Predominant clinical symptoms include motor weakness, dysfunction or spasticity in about 50% of patients, followed by sensory problems and fatigue (40%). About 1.3 million people are currently diagnosed with MS worldwide but estimates are that the total global prevalence is about 2.5 million patients with a male/female ratio of 0.5 (1). The prevalence of MS has a strong geographical pattern, with the highest incidence in Europe (80 per 100.000) and the lowest in Africa (0.3 per 100.000).

Despite considerable research efforts, the underlying causal factors for MS remain largely unknown. The common perception is that MS arises from a combination of genetic and environmental causes. Recent studies have identified a number of risk genes predisposing to MS although the influence of these genes on this disease may be limited since the predictive value of such known risk factors does not exceed 1% (2). That genetic predisposition is not the only factor involved in developing MS is also illustrated by the observations that disease concordance is about 30% for monozygotic twins and that children of MS patients have a 2% chance of getting MS during their lifetime, much higher than the 0.3% background risk for developing MS in the general population (3).

The clinical course of MS is highly variable and difficult to predict for diagnosed patients. MS can manifest itself in three distinct disease patterns. The most common form of MS, approximately 85% of cases at diagnosis, is relapsing-remitting MS (RR-MS), characterized by periods of sudden reduction of neurologic function, followed by partial or complete recovery (remission). Between attacks, patients are free of disease progression. The average age of onset of RR-MS is 29 years (1). Most patients eventually progress to the secondary progressive (SP-MS) phase in which the remission periods no longer occur (3). The transition to the more progressive course of disease occurs in about 50% of the patients within ten years after diagnosis and the percentage steadily increases with disease duration, up to about 80% of patients initially diagnosed with RR-MS. A smaller group of patients (about 10%) experience a continuous progression of clinical symptoms without remissions from the onset (primary progressive or PP-MS).

### 1.2. MS underlying pathology

Under normal conditions, the influx of peripheral leukocytes into the brain parenchyma is restricted by a highly specialized barrier that consists of the endothelial cells (ECs) that line the inner parts of the cerebral microvasculature, the so-called blood-brain barrier (BBB). Therefore, the brain is often referred to as an immune privileged organ. However, this privilege is not absolute and immune surveillance by the adaptive immune system is a normal physiological process, especially close to the ventricles, choroid plexus, meninges and circumventricular organs (4;5). In recent years, it has become clear that, due to the specific immune status of the brain, the immune response in the brain may differ from that in peripheral tissues (6).

In MS, lesion formation is a local phenomenon that occurs predominantly in the white matter of the CNS, mostly near the spinal cord, brain stem, optic nerve and periventricular areas (7). White matter (WM) lesions are inflammatory areas with a local dense infiltration of myelin-loaded (foamy appearing) macrophages accompanied by a variable amount of perivascular and parenchymal T lymphocytes. Usually few B cells are found in active lesions (8). Such lesions can be readily detected using magnetic resonance imaging (MRI), as failure of the BBB allows the ingress of vascular contrast agents at the lesion site. The infiltrating immune cells subsequently cause degradation of the myelin sheaths that line the axons, leading to diminished nerve signal propagation, axonal loss and ultimately severe disability of patients. Active, inflammatory lesions are a hallmark of RR-MS (9), while progressive forms of MS are less defined by acute inflammation. In progressive MS, chronic progressive neurodegeneration is the most prominent pathological feature, which results in diffuse white matter abnormalities and accumulation of axonal loss (10;11).

However, the WM is not the only area affected by the disease as lesions can be detected in the grey matter (GM) as well (12). GM lesions are characterized by activated microglia, demyelination and neurodegeneration, but infiltrating leukocytes are scarcely detected. BBB failure in GM lesions is less prominent, although subtle but persistent BBB malfunctioning has been demonstrated (13). Accumulating evidence suggests that GM atrophy plays an important role in MS pathogenesis and develops early in disease (14;15). Whether these different forms of neuroinflammation are connected or rather independently developing processes is still an active research topic.

### 1.3. The BBB in MS

In MS, the BBB becomes diminished and loses its protective function both in the relapsing remitting and the progressive phases of the disease. Importantly, BBB dysfunction is considered a common denominator in several other neurological conditions such as Parkinson's disease (PD), stroke and epilepsy. A dysfunctioning BBB may contribute to neurological problems that are associated with HIV infection (16) and possibly plays a role in Alzheimers Disease (AD) (17). This raises the question whether BBB failure is a mere consequence of these diverse pathologies or the underlying cause of observed neuropathologies (or both). The BBB is formed by microvascular brain endothelial cells (BMVECs) which are in close contact with surrounding pericytes, astrocytes, neurons and the extracellular matrix; together forming the so-called neurovascular unit (18). Cellular interactions induced by their close proximity are essential to maintain the unique BBB properties of BMVECs.

In contrast to peripheral endothelium, the endothelial lining of the vessels in the brain is not fenestrated, but forms a closed polarized structure that enables selective nutrient uptake and waste product efflux (19). Adjacent ECs are sealed together by tight junction (TJ) proteins in order to limit paracellular influx of blood-borne cells, proteins or small molecules into the brain. Transport of nutrients into the brain parenchyma is closely regulated by specific transporters which provide the brain with essential glucose and amino acids. Efflux of waste or unwanted products from the brain is actively regulated by ATP-binding cassette (ABC) transporters (20) which are highly expressed on the luminal side of BMVECs. Numerous small molecules are substrates for ABC transporters and effluxed from the BMVECs, thereby protecting the brain from potential neurotoxic compounds.

In active MS lesions large numbers of leukocytes migrate across the immune activated BBB. Transendothelial migration of leukocytes may occur via two pathways; paracellular, in which the leukocyte passes between adjacent endothelial cells or transcellular in which the leukocyte travels through the ECs, leaving the junctions intact (21). Leukocyte migration through the BBB is a complex process that is tightly regulated by the interplay of various cell adhesion molecules (CAM), integrins, cytokines and chemokines (22;23). In MS, leukocyte diapedesis most likely occurs at post capillary venules (21;24).

By in vitro studies, it was shown that leukocyte diapedesis negatively influences BBB integrity thereby aggravating BBB failure (25). This observation is supported by studies using

Natalizumab or Alemtuzumab, recently established monoclonal antibody therapies for MS, which demonstrated that blocking T cell infiltration into the brain not only reduces the inflammation but also reduces the number of gadolinium-enhanced lesions dramatically (26). This suggests the involvement of the BBB early in the disease process. Indeed, in the established animal model for MS, experimental allergic encephalomyelitis (EAE), it was shown that BBB leakage precedes leukocyte influx (27;28).

### 1.4. Available treatment

Nowadays, the treatment of MS is aimed at relieving symptoms and reducing disease progression. Disease-modifying drugs (DMD) aim to reduce relapse frequency and accumulated disability in RR-MS. Because MS is considered to be an autoimmune disease, the majority of currently developed DMDs target the immune system. This approach is moderately effective in RR-MS but has little benefit for patients suffering from progressive forms of MS.

The first line of treatment of MS is interferon (IFN)-β (Avonex, Rebif or CinnoVex). IFN-β delays the relapse frequency and shows a slight but significant effect on the accumulation of disability over three years (3). However, this drug has no beneficial effects in the progressive phase of the disease, either primary or secondary. Adverse effects of IFN-β are limited to flu-like reactions. Importantly, about 5-30% of the patients on IFN-β treatment develop neutralising antibodies, thereby reducing treatment efficacy. Another DMD is glatiramer acetate (Copaxone). It is a random polymer (of about 6.4 kDa) made from four amino acids that are present in myelin basic protein (MBP), a structural component of myelin. The exact mechanism of action for glatiramer remains unknown but treatment results in general dampening of the immune response (29). In a long-term study (15 years), it was shown that glatiramer treatment reduces the frequency of relapses in patients with RR-MS, but the number of patients that developed SP-MS was comparable to that of patients on placebo treatment (30).

Mitoxantrone (Novantrone) was originally developed as an anti-cancer drug with its major mechanism of action the inhibition of DNA and RNA synthesis. In MS, treatment of patients with mitoxantrone leads to decreased differentiation of the T cell, B cell and macrophage cell pools, thereby acting as a general immunomodulatory therapy (31). Patients on mitoxantrone experience some beneficial effects, but at the risk of developing severe side effects such as a acute leukaemia and cumulative cardiotoxicity (32). Fingolimod (Gilenya) is a sphingosine -1 –

phosphate (S1P) receptor agonist which limits general leukocyte egress from lymph nodes, thereby reducing numbers levels of circulating leukocytes, limiting leukocyte entry into the CNS (33). Fingolimod reduces both relapse rate (by 54%) and progression to disability (by 30%) in MS patients compared to placebo (34). Teriflunomide (Aubagio) is a new oral anti-inflammatory drug that was approved for treatment of relapsing forms of MS in several regions including Europe, North America, Latin America and Australia (35-37). The active compound is the metabolite of leflunomide. Leflunomide was previously approved for the treatment of rheumatoid arthritis (38) and was shown to possess disease modifying properties in relapsing remitting MS. Although the therapeutic mode of action in MS is not fully understood yet, teriflunomide acts as an inhibitor of dihydroorotate-dehydrogenase (DHODH), an enzyme located in the mitochondria, which is involved in the biosynthesis of pyrimidines. This inhibition leads to a reduction of lymphocytes proliferation and function (39;40). Another new drug which is also used for the treatment of relapsing MS is Dimethyl fumarate (Tecfidera), also know as BG-12. It is a derivate of fumarate which has been used for decades in the therapy of psoriasis (41). Dimethyl fumarate and its main metabolite, monomethyl fumarate, act as modulators of nuclear factor E2-related factor-2 (NRF-2) pathway. This pathway directly regulates the response to oxidative stress and modulates immune response. Although the exact mechanism of action is not fully elucidated yet, dimethyl fumarate shows an inhibitor effect on immune cells as well as on the expression of adhesion molecules and proinflammatory cytokines. It also shows anti-oxidant properties (42). Natalizumab (Tysabri) is a monoclonal antibody against VLA-4 ($\alpha 4\beta 1$ integrin), which is expressed on the surface of lymphocytes. It reduces the relapse rate at 1 year by 68% and the chance of acquiring permanent disability in a two year period by 42% (43). Unfortunately, about 10% of the patients develop neutralising antibodies and in some cases (3.4 per 1000 patients) the use of Natalizumab can cause progressive multifocal leukoencephalopathy (PML), a viral infection of the brain which can be fatal (43). Whether Natalizumab is effective in SP-MS is now being assessed.

Together, the data above indicate that suppressing the immune system is beneficial in reducing the number of relapses and, to some extent, in decreasing accumulated disability in MS. However, this therapeutic approach cannot halt disease progression, nor can it offer relief in progressive forms of MS. In addition, it may lead to serious side effects and, therefore, there remains a high and unmet need for the discovery of more specific drugs aimed at modulating MS disease

progression. As the BBB plays a prominent role in MS disease pathogenesis, preventing BBB failure or accelerating its recovery by restoring inflammation-induced molecular changes may well provide a novel way of modulating disease progression.

### *2. MicroRNAs (miRNAs) are key regulators of protein expression*

Although it has been generally assumed that most relevant genetic information is transmitted by proteins, during recent decades it has become clear that the majority of the genomes of mammals and other complex organisms is in fact transcribed into RNAs that do not encode for protein, so called non-coding RNAs (ncRNAs). The discovery of thousands of ncRNAs has been driven by large-scale genome sequencing, chromatin immunoprecipitation and genome tiling approaches (44;45). Until recently, most of the known ncRNAs (rRNAs, tRNAs and small nuclear and nucleolar RNAs) were implicated in relatively generic processes in a cell ultimately leading to the synthesis of protein. Recent evidence suggests that ncRNA plays an important role in cell biology and orchestrates many physiological and pathophysiological processes in mammalians (for review see (46-52)). In fact, while only 2% of the human genome encodes mRNA and leads to the synthesis of protein, the majority is described as long (>200 nt) and short (<200 nt) ncRNAs. So far, a diverse set of small regulatory RNAs have been identified and characterized including miRNAs, PIWI-interacting RNAs (piRNAs) and promoter associated RNAs (PASRs) and more recently miRNAs that can act as competitive endogenous RNAs (ceRNAs) (53).

MiRNAs are small, about 22 nucleotides, endogenous ncRNA sequences. One of their most important functions is regulation of protein expression by sequence specific binding to the 3' untranslated regions (3'UTRs) of target mRNAs, leading to altered protein expression. Although the first reports on functional short ncRNA sequences (later named miRNAs) in *Caenorhabditis Elegans* stem from the early 1990's (54;55), the first reports linking miRNAs to disease status started emerging from 2006. Since then it was discovered that miRNAs are deregulated in numerous diseases and play a role in many cellular processes. Research on miRNAs as biomarkers, regulators and potential therapeutic targets is still increasing.

The general importance of miRNAs in cellular functioning was demonstrated in experimental developmental studies in which DICER, a protein essential for miRNAs processing, was eliminated. Indeed, loss of miRNAs processing led to abnormalities in cellular differentiation and to embryonic lethality (56;57). To date, over 1800 unique human precursor miRNAs sequences resulting in over 2500 mature miRNAs are annotated in miRbase (58) and still more are being discovered, greatly aided by the rapid development of deep sequencing techniques (59). However, many of these sequences need further functional analysis to determine whether they meet the criteria for miRNAs as described by Ambros and colleagues in 2003 (60).

### 2.1. MiRNAs: biogenesis, degradation and mode of action

MiRNA loci can be found in intergenic regions or in introns, situated alone or in miRNA clusters (61;62). They are transcribed by RNA polymerase II and III, processed by Drosha and Dicer and eventually loaded into the RNA-induced silencing complex (RISC) (62) (Figure 1). The miRNA guides the RISC complex to the target mRNA, where it binds to the 3'UTR region, leading to either destabilisation and degradation of the targeted mRNA or to repression of mRNA translation (63). Whether mRNA degradation or translation inhibition is the dominant mechanism of action is still an active topic of debate (64). Several papers show that significant protein down-regulation is generally accompanied by reduced mRNA levels. The reduction of protein output is modest, up to 50%, leading to the notion that miRNAs are mostly involved in fine-tuning of protein expression levels (65;66). In line with this the recent discovery of miRNAs that has a function as ceRNAs is of high interest (53).

Most mRNA 3'UTRs can bind several miRNA sequences and it has been shown that multiple different miRNAs can target one mRNA. The binding of one miRNA to its target is sufficient to downregulate protein expression significantly, which has been demonstrated in a large number of papers. However, binding of two different miRNAs to one mRNA enhances the repressing effect (67) and a combinational approach may be even more effective. The amount of microRNA that is available for mRNA regulation in the cell is governed by different mechanisms. Apart from the conventional binding of miRNA to mRNA targets, miRNAs can also bind to other RNA species such as circular RNA, long non-coding RNA or pseudogenes. These competitive endogenous RNAs (ceRNA) can bind microRNAs with microRNA response elements and indirectly de-repress miRNA target mRNA (68). In other words, an increase in RNA can sponge up more miRNA, allowing other RNA to act unhindered.

A number of recent studies show that miRNAs can also bind to their target mRNA outside the 3'UTR sequence or to DNA sequences. Indeed, some miRNAs show effective simultaneous 3' and 5' UTR binding (69), or binding on mRNA sequences in the open reading frame (ORF) or on DNA promoter sites of a gene (69;70). In addition, miRNA binding to their target mRNA has been shown in certain cases to lead to mRNA stabilisation, resulting in protein up-regulation (71). Although these findings may open new avenues in the field of miRNA research, they are beyond the scope of this review and will not be discussed here in detail.

Current research is focussed on the biological effects of miRNAs either in health in specific cell types or tissues or during pathogenesis. Yet, relatively little is known about regulation of miRNAs expression by environmental stimuli. MiRNAs are located at diverse positions in the genome. They can be located in introns of protein-coding genes or in introns or exons of ncRNAs. Alternatively, miRNAs can be set between independent transcription units (intergenic) alone or in expression clusters (72). Even miRNAs located in introns have their own transcription regulatory pathways, as only one third of intronic miRNAs are transcribed simultaneously with their flanking gene (73). Thus, regulation of transcription is diverse and not governed by one single mechanism. Although miRNAs are generally considered to be stable RNA sequences, they have diverse half-lives in the cell (74). Some destabilising sequences have been identified, explaining more rapid breakdown for some sequences, but it was also shown that some mature miRNAs have different half-lives under different conditions such as cell cycle stage or stimulation with growth factors that can directly influence miRNA degradation. Several miRNases are known and these findings suggest that miRNAs levels are actively regulated.

### 2.2. Identification of downstream targets of miRNAs

Although there is a fair grasp on the mechanisms of action of some miRNAs, identifying direct targets of miRNAs has proven to be difficult. MiRNAs target prediction databases have been valuable in determining downstream targets, although the predictive value is limited. A recent study comparing different algorithms showed that the average precision of nine different programs was 17,3 % (67). Some programs get higher percentages of accurate predictions but they are significantly less sensitive; they fail to predict a large number of miRNA sequences that were found active.

There are several ways of identifying physiological miRNAs targets, nicely reviewed by Thomson and colleagues (75). Most of them rely on over-expression or inhibition of a target miRNA in a cell line followed by assessing the downstream effects, for example, by transcriptome analysis or by assessing a specific cellular function. An unbiased approach for defining several targets on the protein level can be obtained by using proteomics after miRNAs up- or down-regulation. This is a successful approach, although some caution should be taken as indirect effects are hard to predict (e.g. a miRNA targeting a transcription factor), miRNA expression levels may vary during the cell cycle (76), and over-expression of miRNAs to non-physiological levels may lead to non physiological effects (77). Once putative mRNA targets

have been identified for a miRNA, luciferase assays are commonly used in order to validate this prediction. The 3'-UTR sequence of the target gene is subsequently coupled to the luciferase gene, allowing luminescent readout of miRNA effects. Ideally, additional studies with mutations in the miRNA binding sequences are performed to confirm miRNA involvement, as there are other factors capable of binding at the 3'-UTR as well (78).

## *3. MiRNAs in MS*

MiRNAs can be released into the circulation by activated cells during the pathogenesis of a particular disease. Some of the circulating miRNA are located within exosomes and therefore they are protected from RNase digestion and degradation (79;80). Circulating miRNAs can also be protected from degradation through their association with specific proteins in order to form a protein-miRNA complex such as RISC or Argonaut proteins (81). While MS-associated changes in levels of miRNAs in many different cell type, including peripheral blood mononuclear cells (PBMCs) (82;83), B lymphocytes, CD4+,CD8+ T cell (68), peripheral blood leukocytes (83;84), and brain astrocytes (87) have already been proven, their functions in these cell types remains to be largely understood whereas the role of circulating miRNAs in MS progression has not yet been addressed.

### 3.1. Circulating miRNAs in MS

A recent study showed that in MS a specific circulating miRNAs signature can be recognized (88). ). Six specific miRNAs, miR-614, miR-648, miR-572, miR-422a, miR-1826 and miR-22 were found to be significantly increased in the plasma of MS patients when compared to healthy controls (HC). Importantly, all but one of these miRNAs had not yet been associated with the pathogenesis of MS. Amongst the identified miRNA signature, miR-614 showed the highest fold change between MS patients and HC. This specific miRNA can regulates the expression of a Forkhead transcription factor, FOXD1, which can regulate inflammatory response by suppressing the activation of naïve T cell (89;90). Thus, high levels of miR-614 may lead to T cell proliferation and systemic inflammation (89).

Another novel MS associated miRNA with highly expressed plasma levels in patients compared to control samples was miR-648. A predicted target of this miRNA is the MBP, a CNS-specific myelin protein, which is involved in the stabilization of the myelin sheath in the CNS (91). Once again, over-expression of this miRNA results in a reduction of MBP expression, thereby reducing myelin sheath stability. Furthermore, the expression profile of circulating miRNAs has shown to be different in RR-MS and SP-MS patients compared to HC and in RR-MS versus SP-MS, indicating a specific circulating miRNAs profile linked to disease stage and disability can be recognized (92). These studies together provide evidence of a unique circulating miRNAs signature in MS. Their role in disease pathogenesis remains to be established. However, the

identification of a specific panel of plasma derived miRNAs in different stages of the disease has laid the foundations for further analysis to use miRNAs as a promising biomarker for MS, which is highly sought for.

### 3.2. MiRNAs as biomarker in MS

The establishment of proper markers used to monitor a disease's progression is an essential point for most pathological conditions including cancers, neurodegenerative disease, heart disease and diabetes. Circulating miRNAs possess the characteristics of promising biomarkers, especially since most are known to be stable in the circulation and resistant to RNase digestion and multiple freeze-thaw cycles (79). Moreover, the capability of miRNAs to act as gene regulators, make them potential more suitable as early stage disease biomarkers than proteins and genes.

So far, several circulating miRNAs in plasma have been successfully identified as biomarkers for a number of diseases (93;94). For instance, specific glioma associated miRNAs were identified in serum-derived exosomes from patients affected by glioblastoma. This finding suggests that specific miRNAs released by tumor cells may be useful for diagnostic purpose (95). Additionally, the discovery of miRNAs in human salivary samples suggests a promising use of salivary exosomes as novel and easily accessible biomarkers for disease diagnosis (96).

The function of exosomes as molecular carriers and their ability to delivery miRNAs to cells at a distance, ergo modify target cells gene expression, may further allow their use as diagnostic markers. This evidence allows us to postulate that miRNAs, especially those carried in the blood by exosomes, can be considered as ideal candidates for disease biomarkers. Furthermore, because exosome isolation is a non-invasive procedure and the subsequent characterization of the miRNAs expression profile is nowadays relatively straightforward, exosome profiling represent a great promise as a new diagnostic strategy. Further identification and characterization of exosomal and circulating miRNAs is pivotal in order to establish new miRNAs as biomarkers for diagnostic purposes and, therefore, potentially provide new insights into therapeutic strategies.

### *4. MiRNAs expression profiles in vascular beds in disease state*

The expression profile of miRNAs varies in different tissues, but few miRNAs were found exclusively expressed in specific cell types or tissues (97). In the following section, the effects of miRNAs and their regulation in different vascular beds will be discussed.

#### 4.1. Differential miRNAs expression in ECs

ECs may have different characteristics depending on the surrounding tissue and it is therefore not surprising that the miRNA profile can differ as well. This is illustrated by McCall et al. (98) in a study comparing miRNA expression levels in healthy untreated human endothelial cells isolated from aorta (HAEC), coronary artery (HCEC), umbilical vein (HUVEC), pulmonary artery (HPAEC), pulmonary microvasculature (HPMVEC), dermal microvasculature (HDMVEC) and brain microvasculature (HBMVEC), the cells that form the BBB. In this study, 843 miRNAs were measured and 164 could be detected in ECs. Of these, 40% were differentially expressed in at least one comparison (e.g. HPAEC vs HCEC). Of note, the miRNA profile of HBMVEC showed the closest correlation with that of HUVEC cells instead of the other microvasculature lines. Three miRNAs were significantly different across all EC-types; let-7b, miR-20b and miR-99b. However, whether these three miRNAs (or others) contribute to maintaining the EC phenotype remains to be established.

#### 4.2. The role of miRNAs in endothelial function

Vascular endothelial cells have many functions such as control of angiogenesis, response to inflammation and the regulation of blood coagulation (99). MiRNAs are known to be involved in all of these processes. Upon inflammatory stimuli induced by pro-inflammatory mediators such as cytokines or free radicals, numerous signalling pathways are activated in ECs. A central pathway in endothelial inflammation is NF-κB signalling, which results in the expression of multiple inflammatory genes such as adhesion molecules (P- and E-selectin, vascular cell adhesion molecule (VCAM) , intercellular adhesion molecule (ICAM)) and various cytokines and chemokines (100;101). Many miRNAs that are involved in endothelial inflammation target genes in this pathway (table 1).

An important driver of angiogenesis and endothelial permeability is the vascular endothelial growth factor (VEGF). It has been demonstrated in several studies that VEGF reduces barrier

integrity by signalling through its receptors (102;103). Elevated VEGF levels were found in AD, PD and stroke; all conditions in which the BBB is compromised. It is known that VEGF signalling in ECs is directly influenced by multiple miRNAs among which miR-155 and miR-126 (104). In an attempt to identify miRNAs which regulate the BBB, we recently performed expression analyses in an immortalized human cerebral microvascular endothelial cell (hCMEC/D3) line, hCMEC/D3 cells cultured under barrier-promoting or barrier-disturbing conditions. Using *in vitro* BBB models, we assessed miRNAs expression in hCMEC/D3 that were treated either with pro-inflammatory cytokines or astrocytes conditioned medium (ACM). Pro-inflammatory cytokines reduce barrier functioning, whereas ACM is capable of increasing barrier resistance. We found that under the barrier improving conditions, induced by ACM, many miRNAs were up-regulated. Conversely, treatment that results in lowered barrier resistance, i.e. pro-inflammatory cytokines, is accompanied by decreased miRNA expression levels. In short, we identified a set of microRNAs that are involved in BBB regulation. Within the miRNAs signatures, 50 miRNAs were identified that were regulated by both treatments, i.e. these miRNAs were downregulated in a leaky barrier and upregulated in a tight barrier. In a final set of experiments we wanted to show the relevance of such miRNAs in MS. To this end, we compared the expression of sixteen miRNAs in this specific set in BBB endothelial cells isolated from post-mortem MS lesion areas compared to cells isolated from normal appearing white matter. Strikingly, all selected miRNAs were reduced in the BBB associated with MS lesions. Since subsequent studies showed that forced expression of miRNAs resulted in an increase in BBB function and reduced transendothelial migration of primary monocytes, a hallmark immune cell of MS, our results strongly indicate that therapeutic application of miRNAs could be a viable approach for treating diseases of the CNS which are marked by vascular dysfunction, in particular MS (105). In addition, this highly-specific MS miRNA signature can potentially be used as a biomarker for diagnosis, prognosis and treatment efficacy monitoring.

### 4.3. MiRNAs in BBB functioning

A small number of papers describe miRNAs in BBB functioning in various conditions. Mir-29a is reported to indirectly influence barrier functioning by targeting gene that regulate epigenic events (DNMT genes) and may be a contributing factor in ischemic brain injury (106). In another condition affecting BBB functioning, HIV infection, it was demonstrated that miR-101

expression is increased by the HIV Tat C protein. Mir-101 in turn directly targets VE-Cadherin expression and thus negatively influences barrier functioning (107).

MiR-155 is an extensively studied miRNA which has functions in inflammation (108), autoimmunity and proliferation (109). It is one of the most highly upregulated miRNAs in acute MS lesions (110). It was found that miR-155 is rapidly up-regulated under inflammatory conditions in vitro, has high expression levels in BECs, in the mouse EAE model and miR-155 knockout mice develop less clinical symptoms. MiR-155 exerts its effects by directly targeting DOCK-1, SDCBP, ANXA-2 and Claudin-1 (111), all factors directly relevant to BBB functioning.

Another relevant BBB miRNA is miR-125a-5p. miR-125a-5p is known as a tumour suppressor miRNA (112;113). It also has an anti-inflammatory function in macrophages by inducing the formation of anti-inflammatory type 2 macrophages (114). Moreover, we found that miR 125a-5p is severely reduced in endothelial cells derived from MS lesions and upon inflammatory stimuli in vitro. It directly regulated barrier function in an in vitro BBB model and can reduce monocyte migration through a BBB cell layer in vitro (105).

Future research will be directed on the identification of specific miRNAs profiles that regulate BBB function under different pathological conditions, in order to come to a panel of miRNAs that may be suitable as therapeutic strategies to restore BBB function to prevent disease progression in MS.

## 5. Concluding remarks

MiRNAs are important regulators of protein expression which are involved in many cellular processes. They may prove to be valuable diagnostic markers for a number of diseases and play a yet enigmatic role in intercellular communication. In neuroinflammation, a clear role for miRNAs is emerging as key regulators of endothelial functioning and endothelial response to external stimuli. We and others have shown that miRNA expression levels are altered under inflammatory conditions as seen in MS and potentially several other neurological conditions. Moreover, there are clear indications that restoring miRNA expression levels is beneficial for BBB functioning.

The potential of miRNAs as therapeutic agents is, at this point in time, still rather limited. There are some clear advantages for targeting miR sequences as a therapeutic approach. MiRNAs have several predefined targets which will all be influenced by the same sequence. Therefore miRNAs are regulators of cellular functions by targeting several key genes in a pathway rather than by a single target approach, such as siRNA. The sequence is endogenous and is therefore not likely to elicit immune responses. Moreover, the exact nucleotide sequence of the targeted miRNA is known, limiting lead optimisation to chemical stabilisation and optimisation of delivery.

Despite the relative novelty of the field, two miRNA-based strategies are currently being tested in clinical trials, paving the way for further development of miRNA-based therapeutics. One such a therapy involves Miravirsen, a miR-122 inhibitor used for treating hepatitis C, which showed efficacy in a phase II clinical trial (115). Recently, Mirna Therapeutics started a clinical phase I trial using a miR34a mimic for unresectable primary liver cancer. Based on the outcome of such studies, the use of miRNA-based therapeutic approaches to restore BBB function in MS may become within reach.

## 6. Abbreviations

MS: Multiple sclerosis

CNS: Central nervous system

BBB: Blood-brain barrier

BECs: Brain endothelial cells

miRNA: microRNA

RR-MS: Relapsing-remitting MS

SP-MS: Secondary progressive MS

PP-MS: Primary progressive MS

ECs: Endothelial cells

GM: Grey matter

BMVECs: Microvascular brain endothelial cells

CAM: Cell adhesion molecules

DMD: Disease-modifying drugs

INF-β: Interferon beta

MBP: Myelin basic protein

ncRNAs: Non-coding RNAs

ceRNAs: Competitive endogenous RNAs

UTR: Untranslated region

RISC: RNA-induced silencing complex

HC: Healthy controls

VEGF: Vascular endothelial growth factor

hCMEC/D3: Human cerebral microvascular endothelial cell

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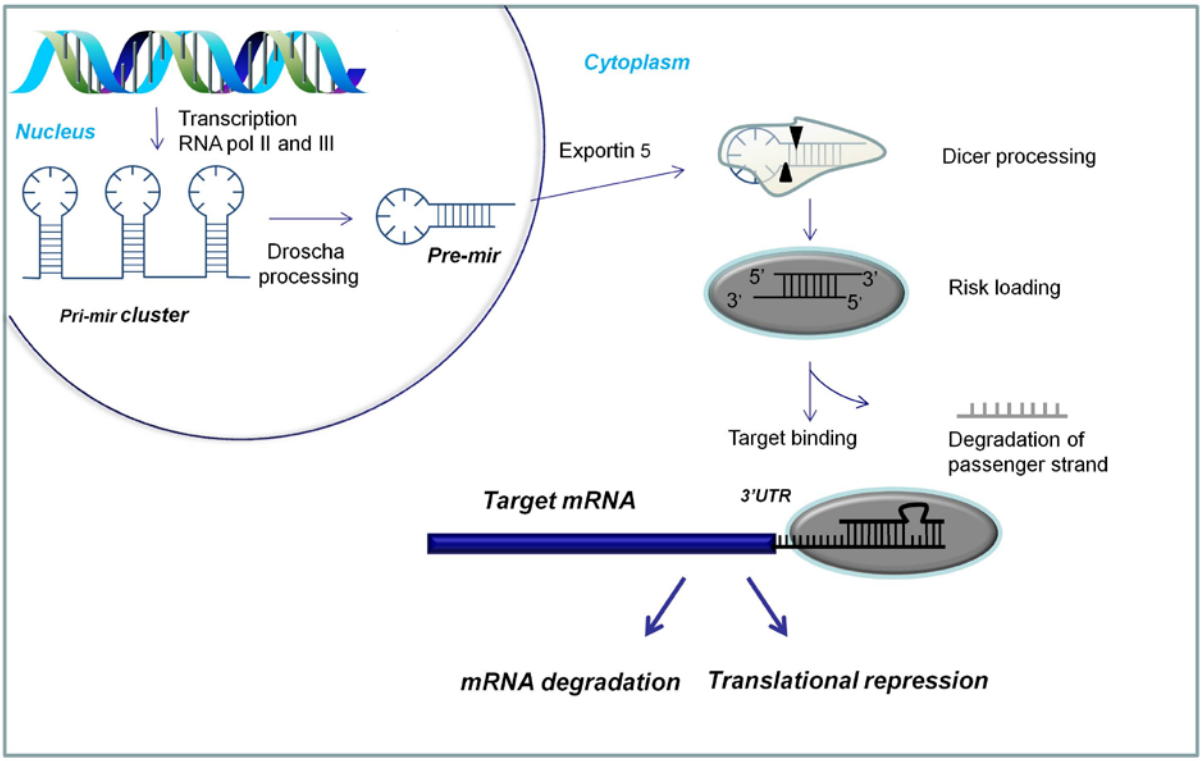


**Figure 1: miRNA biogenesis**.
MiRNAs are transcribed from the genome (sometimes in clusters as depicted) by RNA polymerase resulting in the pri-miRNA. Drosha processes the pri-miRNA to a pre-miRNA which is a 70-100 nt hairpin structure. The pre-miRNA is exported to the cytoplasm by exportin-5 and cleaved by dicer, resulting in an active and a passenger strand. The passenger strand is degraded and the active strand is loaded into the RISC complex. The miRNA guides the RISC complex to the target mRNA leading to translational repression or mRNA degradation

**Table 1: MiRNAs with endothelial functions**

| MicroRNA | Process | Molecular Targets | Reference |
|---|---|---|---|
| Hsa-miR-361-5p | Angiogenesis | VEGF | (116) |
| Hsa-miR-124 | Angiogenesis | Ras | (117) |
| Hsa-miR-125b | Angiogenesis | MAZ | (118) |
| Hsa-miR-146 | Angiogenesis/Inflammation | IRAK1, TRAF6, HuR, TLR4 | (119) |
| Has-miR-126 | Angiogenesis/Inflammation | VCAM, VEGFR2 | (120;121) |
| Hsa-miR-155 | Angiogenesis/Inflammation | VEGFR2/ANXA2, CLDN1, SDCBP, DOCK1 | (101;111) |
| Hsa-miR-125a-5p | Angiogenesis/Inflammation | Endothelin-1/ TJ proteins | (105;122) |
| Hsa-miR-138 | Inflammation | S110A1 | (123) |
| Hsa-miR-712 | Inflammation | TIMP-3 | (124) |
| Hsa-miR-149 | Inflammation | ?, TNF pathway | (125) |
| Hsa-miR-181 | Inflammation | Importin α3 | (100) |
| Hsa-miR-221 | Inflammation | Ets-1 | (126) |
| Hsa-miR-31 | Inflammation | E-selectin | (127) |